AD_____

Award Number: DAMD17-02-1-0726

TITLE: Testing Whether Defective Chromatin Assembly in S-Phase Contributes to Breast Cancer

PRINCIPAL INVESTIGATOR: Peter Adams, Ph.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, PA 19111

REPORT DATE: October 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2004	Annual (30 Sep 2003 - 29 Sep 2004)

4. TITLE AND SUBTITLE
Testing Whether Defective Chromatin Assembly in S-Phase Contributes to Breast Cancer

5. FUNDING NUMBERS
DAMD17-02-1-0726

6. AUTHOR(S)

Peter Adams, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Fox Chase Cancer Center
Philadelphia, PA 19111

E-Mail: Peter.Adams@fccc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Cancer cells characteristically have a high frequency of genome rearrangements. Although these genome rearrangements are likely to contribute to the defective proliferation control that is characteristic of cancer cells, the cause of rearrangements is poorly understood. We used a dominant negative mutant of (chromatin assembly factor-I) CAF1, a complex that assembles newly synthesized DNA into nucleosomes, to inhibit S-phase chromatin assembly and found that this induced S-phase arrest. Arrest was accompanied by DNA damage. These results show, for the first time, that in human cells CAF1 activity is required for completion of S-phase and defects in chromatin assembly induce DNA damage. We propose that errors in chromatin assembly, occurring spontaneously or caused by genetic mutations or environmental agents, contribute to genome instability and cancer. Consistent with this idea, preliminary evidence indicates that chromatin assembly factors are mutated in some human cancers.

14. SUBJECT TERMS
chromatin assembly checkpoint

15. NUMBER OF PAGES
8

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Cancer cells characteristically have a high frequency of genome rearrangements *(1)*. Although these genome rearrangements are likely to contribute to the defective proliferation control that is characteristic of cancer cells, the cause of rearrangements is poorly understood. We used a dominant negative mutant of (chromatin assembly factor-I) CAF1 *(2)*, a complex that assembles newly synthesized DNA into nucleosomes, to inhibit S-phase chromatin assembly and found that this induced S-phase arrest *(3)*. Arrest was accompanied by DNA damage. These results show, for the first time, that in human cells CAF1 activity is required for completion of S-phase and defects in chromatin assembly induce DNA damage. We propose that errors in chromatin assembly, occurring spontaneously or caused by genetic mutations or environmental agents, contribute to genome instability and cancer. Consistent with this idea, in this last funding period, we have obtained evidence that cells have the ability to "sense" non-chromatinized DNA. Also consistent with this idea, we have obtained additional evidence to indicate that chromatin assembly factors are mutated in some human cancers cell lines and human tumors.

Body.

The original tasks are italicized below and addressed individually.

Task 1. To investigate the processes that monitor chromatin assembly in primary HMECs and determine if these monitoring processes are impaired in transformed breast cancer derived cell lines.

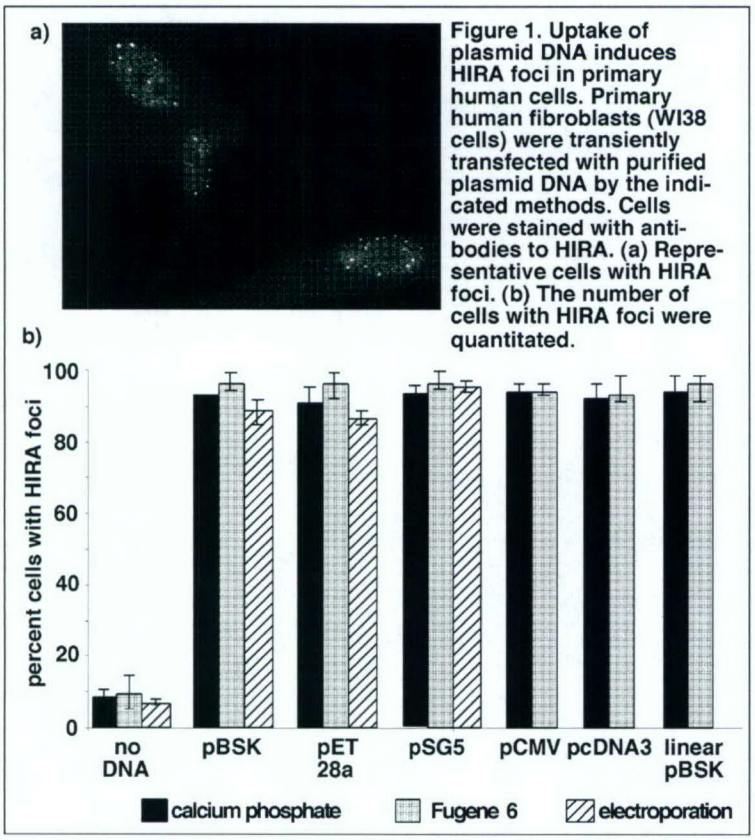

Figure 1. Uptake of plasmid DNA induces HIRA foci in primary human cells. Primary human fibroblasts (WI38 cells) were transiently transfected with purified plasmid DNA by the indicated methods. Cells were stained with antibodies to HIRA. (a) Representative cells with HIRA foci. (b) The number of cells with HIRA foci were quantitated.

In the original application, we proposed that defects in chromatin assembly in S-phase activate a checkpoint that blocks on-going DNA synthesis - a so-called chromatin assembly checkpoint. We subsequently found, in the early stages of DOD funding, that defects in chromatin assembly cause DNA damage *(3)*. A large body of published data has shown that DNA damage causes S-phase arrest *(4)*. Consequently, we now favor the view that the S-phase arrest caused by defects in chromatin assembly is a consequence of DNA damage. This work has been published in *Molecular Cell (3)*. To follow up on this work, we are investigating the mechanism by which cells recognize non-chromatinized, naked DNA. Although the course of this work is a departure from the original application, we feel that good progress is being made. Indeed, data related to those shown here are currently "in press" in *Developmental Cell (5)*.

a. Cells respond to non-chromatinized DNA by recruitment of HIRA to PML bodies.

Since the work published in *Molecular Cell* showed that perturbation of replication-coupled chromatin assembly causes DNA damage *(3)*, we have searched for a way to introduce non-chromatinized DNA into the cell, without simultaneous induction of DNA damage. Significantly, we found that uptake of naked, non-chromatinized DNA by the cell causes relocalization of a replication-independent chromatin assembly factor, HIRA, to PML nuclear bodies. Relocalization of HIRA is independent of DNA sequence, the method of introduction of the DNA into the cell (calcium phosphate or liposome mediated transfection or electroporation) and the conformation of the DNA (supercoiled versus linear). Clearly, the cell can sense the presence of the naked DNA. We hypothesize that the cell detects the non-chromatinized DNA and activates a chromatin assembly pathway to package the DNA into chromatin. Activation of this pathway is reflected in relocalization of HIRA to PML bodies. To investigate this further we will: i) test whether the cell is specifically sensing non-chromatinized DNA by microinjecting cells with naked, non-chromatinized DNA and DNA assembled into chromatin *in vitro*. We predict that the former, but not the latter, will induce relocalization of HIRA. ii) investigate the mechanism by which HIRA is relocalized to PML bodies. iii) compare the ability of HMECs and breast cancer cell lines to sense non-chromatinized DNA, as reflected by relocalization of HIRA.

Task 2. To determine whether mutations in chromatin assembly factors occur in primary human breast cancer and whether defects in chromatin assembly contribute to transformation of primary HMECs in culture.

As reported in this report last year, we began by sequencing cDNAs encoding chromatin assembly factors from various transformed human cell lines. We sequenced cDNAs encoding p60CAF1 from 17 human tumor-derived cell lines (including 2 breast cancer cell lines, MCF7 and MDAMB435). In one of these cell lines (U2OS cells - an osteosarcoma derived cell line), we found a C to T somatic base change that is predicted to result in substitution of P427 with L (P427L). This base change was confirmed by sequencing of the corresponding genomic DNA. Since U2OS cells are derived from a Caucasian individual, as a control we sequenced genomic DNA from 100 normal Caucasians with no cancer history. In all of these, residue 427 was predicted to be P. This argues against the P427L substitution being a polymorphic variant, although we cannot rule out a very rare polymorphism. Significantly, the DNA sequence data showed only a C at this position, and no wild type T. Thus,

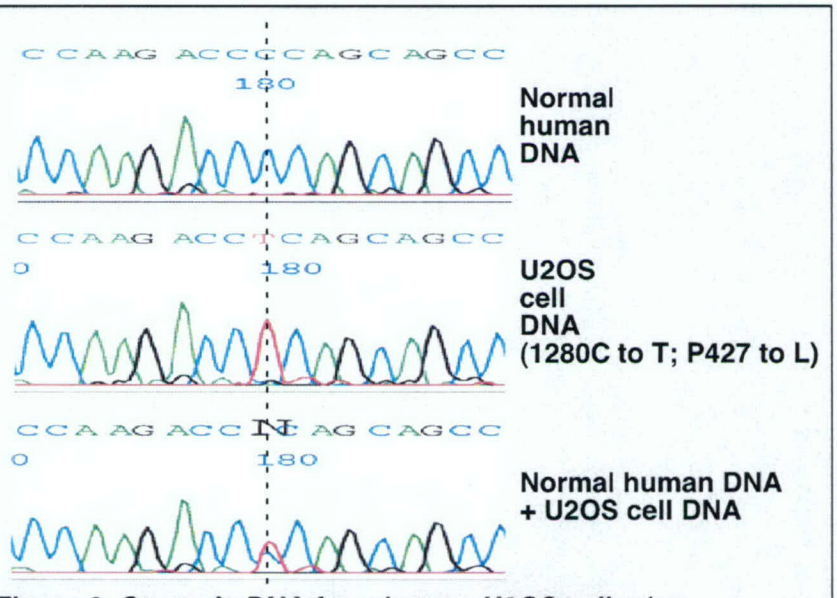

Figure 2. Genomic DNA from human U2OS cells (an osteosarcoma cell line) and normal human DNA was sequenced through the gene encoding p60CAF1A. In the bottom panel the two DNAs were mixed in equal amounts prior to sequencing. A somatic base change in the U2OS cell DNA is indicated by the dashed line.

either U2OS cells are homozygous for the P427L variant, or the 2nd allele is deleted or otherwise not expressed. Since a homozygous, rare polymorphic variant seems highly unlikely, these data support the notion that the P427L variant has been selected for during tumor progression.

We were encouraged by these preliminary data and have continued as described below.

Sequenced cDNAs from primary human tumors.

To date, we have sequenced cDNAs encoding p60CAF1 from 5 human tumors. To do this, total cDNA was prepared from each of 5 fresh frozen tumors. Then, cDNA encoding p60CAF1 was amplified as 5 separate fragments, fragments 1-5. Each of these was direct sequenced, and the sequenced compared with the p60CAF1 sequence deposited in the NCBI database and with known single nucleotide polymorphisms (SNPs, Table 1). Significantly, a single somatic base change, a C to T at position 250, was identified. In the amino-acid sequence, this corresponds to a substitution of alanine to valine. We are currently sequencing the corresponding patient-matched normal human DNA. This will tell us whether this is a sequence polymorphism or a tumor specific mutation. Encouragingly, no sequence polymorphisms are reported at this position.

Table 1: p60CAF1 SEQUENCING RESULTS

Sample ID	**Fragment 1 63-500**	**Fragment 2 451-840**	**Fragment 3 800-1150**	**Fragment 4 1101-1600**	**Fragment 5 1600-2178**
4425	**wt**	**wt**	**wt**	1499 SNP C 1578 SNP A	1958 SNP A/G
4341	**wt**	**wt**	**wt**	1499 SNP C 1578 SNP A	1958 SNP G
4355	**wt**	**wt**	**wt**	1499 SNP C 1578 SNP A	1958 SNP G
4253	**wt**	**wt**	**wt**	1499 SNP C 1578 SNP A	1958 SNP G
4227	**250 C>T**	**wt**	**wt**	1499 SNP C 1578 SNP A	1958 SNP G

Variation	**Position**	**Frequency**
T	**1499**	**.02**
C	**1499**	**.98**
C	**1578**	**.08**
A	**1578**	**.92**
G	**1958**	**.45**
A	**1958**	**.55**

Key Research Accomplishments.

1. This is the first published demonstration that defects in chromatin assembly inhibit DNA synthesis and cause DNA damage in human cells *(3)*. This finding predicts that defects in chromatin assembly will promote the genome instability that is thought to contribute to development of the neoplastic phenotype.
2. We provide evidence that cells can "sense" the presence of non-chromatinized DNA (Figure 1).
3. We provide the first preliminary evidence that S-phase chromatin assembly factors, such as CAF1, are mutated in human cell lines and tumors (Figure 2 and Table 1).

Reportable Outcomes.

Manuscripts.

Prior to 2004, we had already reported key research accomplishments in *Molecular Cell (3)* and this work has been also been extensively reviewed elsewhere *(6-8)*. Other work concerning the role of HIRA as a replication-independent chromatin assembly factor is currently "in press" in the prestigious peer reviewed journal *Developmental Cell (5)*.

Meeting abstracts/presentations

Oral presentation at 2002 Cold Spring Harbor Cell Cycle meeting, Cold Spring Harbor, NY
Poster at 2003 Enzymology of Chromatin and Transcription Meeting, Big Sky, MT
Poster at 2003 Alan Wolffe Chromatin Meeting
Poster presentation at 2003 FASEB Chromatin meeting, CO
Oral presentation at 2003 22nd PSU Molecular Biology Symposium
Oral presentation at 2004 Keystone Cell Cycle and Development Mtg, Snowbird, Utah
Oral presentation at 2004 Cold Spring Harbor Cell Cycle meeting, Cold Spring Harbor, NY
Oral presentation at 2004 Cold Spring Harbor Cancer Genetics meeting, Cold Spring Harbor, NY
Poster presentation at 2004 Cold Spring Harbor Chromatin symposium, Cold Spring Harbor, NY

Invited seminars

2002 University of Pennsylvania, Philadelphia, PA
2003 Cancer Research UK
2003 Dana Farber Cancer Institute, Boston, MA
2003 UC Berkeley, CA.
2004 University of Toronto, Toronto,

Conclusions.

As reported in *Molecular Cell (3)*, defects in chromatin assembly can contribute to DNA damage and genome instability in human cells. Consequently, to maintain genome stability cells must faithfully incorporate all newly replicated DNA into chromatin and/or assembly non-chromatinized DNA into chromatin in a replication-independent manner. Consistent with the latter idea, we have shown that uptake of plasmid DNA into cells triggers relocalization of a replication-independent chromatin assembly factor, HIRA. In addition, preliminary evidence from sequence analysis of chromatin assembly factors in human tumor derived cell lines and human tumors indicates that mutations in chromatin assembly factors might be selected for and drive progression of human tumors. Continuation of this project has the potential to uncover a novel class of tumor suppressor and oncogenes that might, ultimately, provide new diagnostic, prognostic and therapeutic targets.

References.

1. Lengauer, C., Kinzler, K.W., and Vogelstein, B., (1998) Genetic instabilities in human cancers. *Nature*, 396(6712), 643-649.
2. Tyler, J.K., (2002) Chromatin assembly. *Eur J Biochem*, 269(9), 2268-2274.
3. Ye, X., Franco, A.A., Santos, H., Nelson, D.M., Kaufman, P.D., and Adams, P.D., (2003) Defective S-phase chromatin assembly causes DNA damage, activation of the S-phase checkpoint and S-phase arrest. *Molecular Cell*, 11, 341-351.
4. Zhou, B.B. and Elledge, S.J., (2000) The DNA damage response: putting checkpoints in perspective. *Nature*, 408(6811), 433-439.
5. Zhang, R., et al., (2004) Formation of macroH2A-containing senescence associated heterochromatin foci (SAHF) and senescence driven by ASF1a and HIRA. *Developmental Cell*, In press.
6. Ye, X. and Adams, P.D., (2003) Coordination of S-phase events and genome stability. *Cell Cycle*, 2(3), 185-187.
7. Verreault, A., (2003) Histone deposition at the replication fork: a matter of urgency. *Mol Cell*, 11(2), 283-284.
8. Heinrichs, A., (2003) A fork load. *Nature Reviews: Mol Cell Biol*, 4(4), 253.